IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	J. R. NEESER et al.	Confirmation No:	6562
Application No.:	09/779,596	Group Art Unit:	1652
Filing Date:	February 9, 2001	Examiner:	Kathleen Kerr
For:	INCORPORATION OF EXOGENOUS LACTIC BACTERIA INTO THE ORAL MICROFLORA	Attorney Docket No.:	88265-406

SUBMISSION OF PRIORITY DOCUMENT

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Sir:

Applicant has claimed priority under 35 U.S.C. § 119 to European Patent Application No. 98202707.0, filed on August 12, 1998. In support of this claim, a certified copy of this application is submitted herewith.

No fee or certification is believed to be due for this submission. Should any fee be required, however, please charge such fee to Winston & Strawn LLP Deposit Account No. 50-1814.

Respectfully submitted,

6/17/04
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202-371-5904

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The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

98202707.0

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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R C van Dijk

Anmeldung Nr:
Application no.: 98202707.0
Demande no:

Anmeldetag:
Date of filing: 12.08.98
Date de dépôt:

Anmelder/Applicant(s)/Demandeur(s):

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Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

Incorporation of exogenous lactic bacteria into the oral microflora

In Anspruch genommene Priorität(en) / Priority(ies) claimed /Priorité(s) revendiquée(s)
Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation/International Patent Classification/
Classification internationale des brevets:

A23C9/123

Am Anmeldetag benannte Vertragstaaten/Contracting states designated at date of filing/Etats contractants désignées lors du dépôt:

AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE

INCORPORATION OF EXOGENOUS LACTIC BACTERIA INTO THE ORAL MICROFLORA

The present invention relates to the incorporation of exogenous lactic bacteria into the oral microflora intended for the prophylaxis or the treatment of dental caries, dental plaque and periodontal infection.

Background of the invention

The mouth (oral cavity) contains a resident and a non-resident microflora. The first includes microorganisms that are able to establish a more or less permanent residence on the oral surfaces. These bacteria are mainly localised on the tongue, the buccal mucosa and the teeth while the gingiva, lips, cheeks, palate and floor of the mouth only support a very sparse microflora.

On the tongue and the buccal mucosa, the natural resident microflora includes microorganisms selected from Streptococcus, Veillonella, Bacteroides and Haemophilus. On the teeth, Streptococci, Lactobacilli and Actynomyces predominate but a variety of Gram positive and negative cocci and rods can be found.

For example, Frandsen *et al.* showed that *S. sanguis* predominates on the buccal mucosa but its primary habitat is the surface of teeth, that *S. gordonii* grows in the mature supragingival plaque, that *S. oralis* and *S. mitis* grow in the initial dental plaque (Oral Microbiol. Immunol., 6, 129-133, 1991). Strains belonging to the mutans group are localised on teeth (*S. criscetus, S. downei, S. ferus, S. macacae, S. mutans, S. rattus, S. sobrinus*). Strains belonging to the S. milleri group predominate in dental abscesses (*S. anginosus, S. constellatus, S. intermedius*; Bentley *et al.*, Int. J. System. Bacter. 1991, 41, 487-494; Wood *et al.*, The Genera of Lactic Acid Bacteria, Blackie Academic and Professional, Chapman & Hall, W. H. eds., 1995).

Many of these microorganisms are innocuous commensal, but a lot of them have been recognised as the etiologic agent of quite a few diseases (Hill, M. J. and Marsh, P. D. eds. Human Microbial Ecology, 1990, CRC Press, Boca Raton Florida, USA)

The dental plaque is a film that forms on the surface of teeth consisting of bacterial cells in a matrix of extracellular polysaccharides and salivary products. Immediately after eruption, the teeth are covered with an amorphous layer of saliva, the acquired enamel pellicle (AEP) that is about 1.3 μm thick and cannot be removed by normal tooth brushing. The deposition of bacteria on teeth follows immediately the formation of the AEP and plaque becomes evident in 8-12 hours as a multi-layered structure. The first layer consists of bacteria (earliest colonisers) that attach to teeth mainly via specific adhesin-receptor recognition; it forms a substratum for the second colonisers that adhere one to the other via analogous specific binding or via simple juxtaposition. Plaque cohesion is essentially guaranteed by three mechanisms: the presence of a salivary pellicle on the outer bacteria layer, the specific coaggregation among the different bacterial species and the glucans synthesised by the bacteria and that remain entrapped in the plaque matrix (Skopek *et al.*, Oral Microbiol. Immunol., 9, 19-24, 1994; Kolenbrander et al., Meth. Enzymol., 253, 385-397, 1995; Hiroi *et al.*, FEMS Microbiol Lett., 96, 193-198, 1992; Gibbons *et al.*, Infect. Immun., 52, 555-561, 1986).

The organic acids produced by oral bacteria during the fermentation process directly cause dental caries. These acids attack the hard tissue of teeth with consequent release of ions such as calcium, phosphate, carbonate, magnesium, fluoride, sodium. When the pH in the oral cavity increases again around the neutrality, saliva becomes saturated with calcium and therefore its liberation from the tooth is prevented.

Among all the food residues found in the mouth, carbohydrates show the highest caries promoting effect being directly available for oral bacteria fermentation.

Potentially all micro-organisms fermenting sugars are cariogenic, but the primary etiological agents of coronal and root caries are the mutans streptococci because they are strong acid producers; lactobacilli, that are highly aciduric, can also be implicated. In humans, *S. mutans* and *S. sobrinus* are the more cariogenic strains, and live on teeth while not colonising the entire dentition. A decrease in their number from molar to anterior teeth was indeed demonstrated (Lindquist *et al.*, Dent. Res., 69, 1160-1166, 1990). Moreover in human approximal plaque, *S. mutans* and *S. sobrinus* preferentially colonise the most caries-prone site apical to the contact area (Ahmady *et al.*, Caries Res., 27, 135-139, 1993). A higher

prevalence of *S. sobrinus* was also found in the molar regions compared with that of *S. mutans* (Lindquist *et al.*, Caries Res., 25, 146-152, 1991).

S. mutans and *S. sobrinus* have been shown to attach to the pellicle of teeth mainly via specific adhesin-receptor. Gibbons *et al.* showed that *S. mutans* carries an adhesin which binds to salivary components in the pellicle, while *S. sobrinus* cells appear to possess an adhesin which binds to glucan in the pellicle (Infect. Immun., 52, 555-561, 1986).

The transient microflora comprises exogenous bacteria that can be occasionally present in the mouth, but that do not establish a permanent residence (even if repeated oral administrations of these bacteria are carried out). All the food bacteria, and in particular lactic acid bacteria, can be part of this transient microflora. These exogenous lactic bacteria have never been shown to be capable of directly adhering to the pellicle of teeth. Repeated administration of exogenous lactic bacteria may however lead to colonisation of the mouth on all the oral surfaces, such as the tongue, the buccal mucosa, the gingiva, lips, cheeks, palate, floor and the teeth. This colonisation may result from attachments via specific bindings to bacteria of the resident microflora (co-aggregation phenomena), or via entrapment in the matrix of polysaccharides produced by the resident bacteria, or via adhesion to saliva proteins (especially glycoproteins).

Lactobacillus casei rhamnosus GG (ATCC53103) has been reported to colonise the mouth, most probably on the epithelium of the buccal mucosa since this strain also adheres to the epithelium of the intestinal tract (US5032399, Gorbach *et al.*; Micr. Ecol. In Health and Dis., 7, 295-298, 1994). By contrast *L. rhamnosus* does not adhere to teeth.

Japanese patent n°4021633 (Cyconmedix KK) also reported colonisation of the mouth by *Lactobacillus acidophilus*, most probably on the epithelium of the buccal mucosa since many *Lactobacillus acidophilus* are known to adhere also to the epithelium of the intestinal tract (EP577904; EP199535; Perdigon *et al.*, Medicina, 46, 751-754, 1986; Perdigon *et al.*, Immunology, 63, 17-23, 1988).

Exogenous lactic bacteria can also produce factors that inhibit the growth of the resident microflora in the mouth. For example, EP759469 (Société des Produits Nestlé) described the use of a bacteriocin produced by *Micrococcus varians* for inhibiting the development of the oral pathogens *S. sobrinus, S.*

sanguis, S. mutans and *A. viscosus*. Likewise, Meurman *et al.* showed also that the *Lactobacillus casei rhamnosus* GG exerts inhibitory activity against a variety of bacterial species of the mouth, due to the production of a bacteriostatic substance (Eur. J. of Oral Sci., 103, 253-258, 1995).

Some strategies have been used to minimise the development of the resident microflora of the mouth, namely an administration of commensal bacteria of the resident microflora that are not cariogenic, such as *Streptococcus salivarius* and/or *Stomatococcus mucilaginosus,* and/or repeated administrations of exogenous lactic bacteria such as *L. casei, L. fermentum, L. acidophilus, L. crispatus, L. gasseri, L. salivarius, L bulgaricus* and *S. salivarius* (Tanzer *et al.*, Infec. and Immunity, 48, 44-50, 1985; WO92/14475).

The application of bacteriocins is also one of the investigated strategies which have been set up to reduce tooth caries. These molecules have attracted interest as prospective anticaries agents and as factors important in modulating colonisation of the oral cavity. The anti-caries potential of applications of some bacteriocins comes from their potent and broad antibacterial activity against mutans streptococci and bacteria associated with dental plaque, and their natural occurrence in bacteria regarded as human safe (US5368845 of Colgate, and WO94/12150 of Smithkline Beecham).

The application of milk derivatives is also of interest for the health of the mouth. Indeed, US5427769 (Nestec S.A.) describes another alternative where dental caries are prevented by contacting teeth with an edible composition containing micellar casein in amount sufficient to inhibit colonisation by *Streptococcus sobrinus*. EP748591 (Société des Produits Nestlé S.A.) also reports the use of fluoridates micellar casein or its micellar subunits for treating dental caries or plaque. US4992420 (Nestec S.A.) describes treatment of the buccal cavity with kappa-caseino-glycomacropeptide derived from milk for eradicating plaque and caries.

Lactic bacteria that are not part of the resident microflora of the mouth have never been shown to be really capable of directly adhering to the pellicle of teeth. Thus, by colonising the surface of teeth, such lactic bacteria would exert an inhibitory activity against the growth of the resident microflora, including oral pathogens.

Summary of the invention

The object of the present invention is to provide the use of lactic bacteria that is not part of the resident microflora of the mouth, that is low acidifying and that is capable of adhering directly to the pellicle of the teeth, for the preparation of a composition intended for the prophylaxis or the treatment of dental caries, dental plaque and periodontal infection.

Another object is the use of the lactic bacteria that has been genetically modified to increase its adherence to the pellicle of the teeth via adhesion factors and/ or genetically modified to be even less acidifying contributing to a pH in the oral cavity of about 5.5-7.

Another object is the use of lactic bacteria that is not part of the resident microflora of the mouth, for the preparation of a composition intended for the prophylaxis or the treatment of dental caries, dental plaque and periodontal infection, wherein the lactic bacteria is selected from the group consisting of :

- an acidifying lactic bacteria that adheres to the pellicle of the teeth and that has been genetically modified so that it is low acidifying;
- a non adherent lactic bacteria that is low acidifying and that has been genetically modified so that it adheres to the pellicle of the teeth;
- a non-adherent acidifying lactic bacteria that has been genetically modified so that it adheres to the pellicle of the teeth and genetically modified so that it is low acidifying.

Another object is to provide a composition for the health of the mouth comprising a lactic bacteria that is not part of the resident microflora, that is low acidifying and that is capable of adhering directly to the pellicle of the teeth.

Another object is to provide a composition for the health of the mouth comprising (1) at least a lactic bacteria that is not part of the resident microflora of the mouth, which is capable of adhering directly to the pellicle of the teeth and contributing to a pH in the oral cavity of above 5.5, and (2) any forms of caseino-glycomacropeptide, micellar casein, fluorinated micellar casein, rennet milk or bacteriocin.

Another object is to provide a composition for the health of the mouth comprising at least one lactic bacteria strain selected from the group consisting of the strains CNCM I-1984, CNCM I-1985, CNCM I-1986, and CNCM I-1987.

Another object is to provide a composition for the health of the mouth comprising at least one lactic bacteria that has been genetically modified to increase its adherence to the pellicle of the teeth via adhesion factors and/ or genetically modified to be even less acidifying and contributes to a pH in the oral cavity of about 5.5-7.

Another object is to provide a composition for the health of the mouth comprising lactic bacteria selected from the group consisting of :

- an acidifying lactic bacteria that adheres to the pellicle of the teeth and that has been genetically modified so that it is low acidifying;
- a non adherent lactic bacteria that is low acidifying and that has been genetically modified so that it adheres to the pellicle of the teeth;
- a non-adherent acidifying lactic bacteria that has been genetically modified so that it adheres to the pellicle of the teeth and genetically modified so that it is low acidifying.

In a last further aspect, the invention provides a method for screening lactic bacteria capable of adhering to tooth, comprising the steps of: (1) preparing monoclonal antibody recognising specific surface proteins of a lactic bacteria strain capable of adhering to the teeth, (2) screening any lactic bacteria strain by use of the monoclonal antibody of strain capable of adhering to the teeth.

Detailed description of the invention

Within the following description, the mouth is the oral cavity composed by the oral mucosa (gums, lips, cheeks, palate and floor of the mouth), the tongue and the teeth (including artificial structures).

The resident microflora of the mouth includes all microorganisms that naturally live in the mouth because they can establish a permanent residence on the oral surfaces. The resident microflora of the mouth also includes bacteria that live in the interfacial region between the dental hard and soft tissues (the junction tooth-gingiva), even thought the gingival crevice and the periodontal pocket are

not present in a healthy mouth. This microflora includes microorganisms selected from *Streptococcus, Staphylococcus, Enterococcus, Micrococcus, Peptostreptococcus, Peptococcus, Lactobacillus, Corynebacterium, Actinomyces, Arachnia, Rothia, Alcaligenes, Eubacterium, Propionibacterium, Bifidobacterium, Bacillus, Clostridium, Neisseria/Branhamella, Veillonella, Enterobacteriaceae, Campylobacter, Eikenella, Actinobacillus, Capnocytophga, Haemophilus, Simonsiella, Bacteroides, Fusobacterium, Porphyromonas, Prevotella, Leptotrichia, Wolinella/Selenomonas, Mycoplasma, Candida, Spirochaetes*, Protozoa.

The transient microflora comprises exogenous bacteria that can be occasionally present in the mouth, but that do not establish a permanent residence. This transient microflora may comprise all the food micro-organisms, such as the bifidobacteria (*B. infantis, B. adolescentis, B. breve* and *B. longum)*; the lactococci (*Lactococcus lactis* subsp. *lactis, Lactococcus lactis* subsp. *cremoris,* and *Lactococcus lactis* subsp. *lactic biovar diacetylactis);* the streptococci (*Streptococcus thermophilus, S. lactis, S. lactis cremoris* and *S. lactis diacetylactis*); the lactobacilli (*Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus helveticus, Lactobacillus farciminis, Lactobacillus alimentarius, Lactobacillus casei* subsp. *casei, Lactobacillus delbruckii* subsp. *lactis, Lactobacillus sake, Lactobacillus curvatus, Lactobacillus fermentum* and the acidophile group comprising *L. johnsonii*; see Fujisawa et al., Int. J. Syst. Bact., 42, 487-491, 1992); the pediococci (*Pediococcus pentosaceus, Pediococcus acidilactici* and *Pediococcus halophilus);* the enterococci; the staphilococci (*Staphylococcus xylosus* and *Staphylococcus carnosus*); the micrococci (*Micrococcus varians);* yeast of the genus *Debaromyces, Candida, Pichia, Torulopsis* and *Saccharomyces;* and mold of the genus *Aspergillus, Rhizopus, Mucor* and *Penicillium.*

With respect to the first object of the present invention, the use of lactic bacteria that is not part of the resident microflora of the mouth, that is low acidifying and that is capable of adhering directly to the pellicle of the teeth, for the preparation of a composition intended for the prophylaxis or the treatment of dental caries, dental plaque and periodontal infection, is concerned.

The lactic bacteria according to the invention are low acidifying and they are capable of adhering directly to the pellicle of the teeth so that the compositions prepared with these lactic bacteria are intended for deplacing

pathogens of the teeth or preventing their attachment. Lactic bacteria according to the invention are "low acidifying", which means that they are less acidifying than pathogenic strains. Accordingly, they contribute to a pH in the oral cavity of about 5.5-7. They are preferably from dairy origin.

The lactic bacteria according to the invention adhere to the pellicle of the teeth via especific interaction and/or adhesion factors. These adhesion factors are proteins or polysaccharides.

At least one lactic bacteria is selected from the group consisting of *Streptococcus thermophilus*, *Lactococcus lactis subsp. lactis*, and *Lactococcus lactis subsp. lactis biovar diacetylactis* and particularly from the group consisting of the strains CNCM I-1984, CNCM I-1985, CNCM I-1986 and CNCM I-1987.

These strains have been selected among latic bacteria strains for their capacity of adherence to the pellicle of the teeth, their optimal growth temperature is about 37°C, which is the temperature in the oral cavity. Moreover they are capable of fermenting glucose and sucrose and do not synthesise glucans, which are factors of pathogenicity of the cariogenic strains.

According to another object of the present invention, it is considering modifying genetically lactic bacteria so that it adheres to the pellicle of the teeth via adhesion factors. For lactic bacteria that already adheres to the pellicle of the teeth, this modification intends to make the strains more adherent to the surface of the teeth. In the same way, any non-adherent lactic acid bacteria (not Lactobacilli) can be genetically modified so that it adheres to the pellicle of the teeth.

This modification of the lactic bacteria can be achieved by insertion of the genes X17390, X14490 or X53657 (GenBank accession numbers), for example. These gene are responsible in *S. mutans* for the expression of the Antigen I/II that mediates adhesion to salivary glycoproteins.

According to the invention, it is also possible to genetically modify lactic bacteria so that they are low acidifying. For lactic bacteria that is already low acidifying this modification is intended to increase the said effect by decreasing its lactic acid production.

This modification can be achieved in many ways and preferably according to one the protocols described in the following documents : Boumerdassi *et al.*, Appl. Environ. Microbiol., 63, 2293-2299, 1997; Platteeuw *et al.*, Appl. Environ.

Microbiol, 61, 3967-3971, 1995; Ito *et al.*, Biosci. Biotechnol. Biochem., 58, 1569-1573, 1994.

According to the present invention, at least one lactic bacteria genetically modified or not, is used in an "effective quantity" for the preparation of compositions intended for the prophylaxis or the treatment of dental caries, dental plaque and periodontal infection. This quantity is preferably comprised between 10^4 to 10^9 cfu/g.

It is also possible to use at least a lactic bacteria, in combination with milk derivatives, such as milk or fermented milk or milk derivatives selected from any forms of caseino-glycomacropeptide, micellar casein, fluorinated micellar casein, rennet milk (e.g. cuajada) or bacteriocin, for example.

BIOCHEMICAL CHARACTERIZATION OF THE SELECTED STRAINS

Fermentation patterns: 49 simple sugars were tested with the api 50 CH bioMerieux strip test (bioMérieux SA, 69280 Marcy-l'Etoile, France).

Acidification curves: the acidification curves were determined at 37°C in the following conditions:
- *S. sobrinus* OMZ 176: FUM sucrose 1% and FUM glucose 1%
- *S. thermophilus* NCC1561: Belliker sucrose 1% and Belliker glucose 1%

Inoculation was always 5%; pH was recorded every 20 min.

BIOCHEMICAL CHARACTERIZATION OF THE SELECTED STRAINS

Fermentation patterns: 49 sugars were tested with the api 50 CH BioMeriux strep method and the results are given in the table 1.

Acidification curves: *S. thermophilus* NCC1561, from sucrose fermentation, lowers the pH to 4.5, while *S. sobrinus* OMZ 176 to 4.

Sugar	*L. lactis* NCC2225	*L. lactis* NCC2211	*S. th.* NCC1529	*S. th.* NCC1561
Adonitol	+++			
Aesculin	++	++++		
Amygdalin	++++			
D-Arabinose				
L-Arabinose				
D-Arabitol				
L-Arabitol	+++			
Arbutin	+++	+++		
Cellobiose	+++	++++		
Dulcitol				
Erythritol				
D-Fructose	+	++++		
D-Fucose				
L-Fucose				
Galactose	++	++++		
β-Gentiobiose		+++		
Gluconate				
2-keto-Gluconate				
5-keto-Gluconate				
GlcNAc	+	++++		
D-Glucose	+	++++	+	++
Glycerol				
Glycogen				
Inositol				
Inulin				
Lactose	+	++++	+++	++++
D-Lyxose				
Maltose	++			
Mannitol	+++	++		
D-Mannose	+	++++		
Melezitose				
Melibiose				
α-Methyl-D-glucoside				
α-Methyl-D-mannoside				
D-Raffinose				

Rhamnose				
Ribose	++	++		
Salicin	+++	+++		
Sorbitol				
L-Sorbose				
Starch				
Sucrose			+++	++++
D-Tagatose				
Trehalose	++			
D-Turanose	++			
Xylitol	+++			
D-Xylose				
L-Xylose				
β-methil-xyloside				

+, ++, +++, ++++ show if the fermentation beguns after 3, 6, 24 or 48 hours.

Table 1. Sugar fermentation of *L. lactis* NCC2225, *L. lactis* NCC2211, *S. thermophilus* NCC1529 and , *S. thermophilus* NCC1561.

The second main object of the present invention concerns a composition for the health of the mouth comprising a lactic bacteria that is not part of the resident microflora of the mouth, that is low acidifying and that is capable of adhering directly to the pellicle of the teeth.

These compositions are in particular intended for the prophylaxis or the treatment of dental caries, dental plaque and periodontal infection.

The said lactic bacteria strain is selected from the group consisting of *Streptococcus thermophilus*, *Lactococcus lactis subsp. lactis*, and *Lactococcus lactis subsp. lactis biovar diacetylactis* and preferably from the group consisting of the strains CNCM I-1984, CNCM I-1985, CNCM I-1986, and CNCM I-1987.

In such compositions for the health of the mouth, lactic bacteria strains may be genetically modified as described above.

The said lactic bacteria strains may be included in a food, pet food, cosmetic or pharmaceutical composition, for example.

Accordingly, these compositions are preferably toothpaste, mouth rinse, gum, spray, beverage, candies, infant formula, ice cream, frozen dessert, sweet salad dressing, milk preparations, cheese, quark, yogurt, acidified milk, coffee cream or whipped cream, for example.

In these compositions lactic bacteria strains may be included alone or in combination with milk derivatives, for example, in order to obtain synergistic preparations. Accordingly, these compositions for the health of the mouth comprise:

- a lactic bacteria that is not part of the resident microflora of the mouth, which is capable of adhering directly to the pellicle of the teeth;
- any forms of lactic glycopeptides, rennet milk or bacteriocin.

The lactic glycopeptides are preferably caseino-glycomacropeptides (CGMP, Asyalo-CGMP), fluorinated or not micellar casein (which can be obtained as described in the patents EP 0 604 802 and EP 0 748 591) or rennet milk (cuajada for example) may also be added. The caseino-glycomacopeptides are preferably added in a minimum amount of about 0.1%. It has also been shown that the caseino-glycomacropeptides do not prevent the said lactic bacteria from adhering to the teeth pellicle (fig.2 and 3).

Synergistic compositions may also be prepared, adding at least one bacteriocin, which is active against Gram-positive oral bacteria. In that case, the oral hygiene compositions may comprise 0.00001 to 50%, and preferably from 0.00001 to 15% of purified bacteriocin, by weight of the composition. The bacteriocin is preferably variacin (EP 0 759 469).

In order to protect the composition from degradation, an oil-soluble antioxidant may also be included. Suitable antioxidants include the "tocopherols", butyl-hydroxyanisole (BHA), butyl-hydrxytoluene (BHT), and ascorbyl palmitate. The oil soluble antioxidant is present in amounts of from 0.005% to 0.5%, preferably 0.005% to 0.01% by weight of the composition.

Suitable abrasives for use in dentifrice compositions of the present invention include calcium carbonate, calcium aluminosilicate, alumina, hydrates alumina, zinc orthophosphate, plastic particles, and silica, of which silica is the preferred abrasive.

Compositions according to the invention will have a pH which is orally acceptable and within which the activity of the said lactic bacteria is not compromised. The pH may be in the range 3.0-9.5, preferably in the range 3.5 to 6.5.

These compositions may be prepared by conventional processes comprising admixing the ingredients together in the appropriate relative amounts and finally, and if necessary, adjusting the pH to desired value.

According to the last aspect of the present invention, a method for screening lactic bacteria capable of adhering to tooth is concerned. This method comprises the steps of:

(1) preparing monoclonal antibodies recognising specific surface proteins of a lactic bacteria strain capable of adhering to the teeth, and

(2) screening any lactic bacteria strain by use of the monoclonal antibody of strain capable of adhering to the teeth.

The said monoclonal antibodies would be used as a tool to detect the said lactic bacteria strain among other strains growing nearby.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention, in addition to those described herein, will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the claims. Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties to the extent necessary for understanding the present invention. DNA manipulation, cloning and transformation of bacteria cells are, except where otherwise stated, carried out according to the textbook of Sambrook *et al.* (Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, U.S.A., 1989). These examples are preceded by a brief description of the plasmids, strains and the various media used, as well as the method for producing a monoclonal antibody.

The strains *S. thermophilus* S118 (NCC 1529), S123 (NCC 1561), *L. lactis subsp. lactis* 29 (NCC 2211), *L. lactis subsp. lactis biovar dioacetylactis* 69 (NCC 2225) were deposited under the Budapest Treaty, at the Collection Nationale de Culture de Microorganismes (CNCM I-1984, CNCM I-1985,

CNCM I-1986 and CNCM I-1987 respectively), 25 rue du docteur Roux, 75724 Paris, France, on March 3rd, 1998. All restrictions as to the availability of these deposits will be withdrawn upon first publication of this application or another application which claims benefit of priority to this application.

Figures

- Figures 1a, 1b and 1c represent respectively the adhesion saturation curves for *S. sobrinus* OMZ 176, *L. lactis* NCC2211 and *S. thermophilus* NCC1561.
- Figure 2 represents the curves obtained for the three strains by plotting the number of bound cells versus increasing amounts of CGMP.
- Figure 3 represents the curves obtained for the three strains by plotting the number of bound cells versus increasing amounts of As-CGMP.

Example 1: STRAINS AND CULTURE CONDITIONS

More than 100 strains (belonging to the Nestlé culture collection) were screened for their ability to attach to saliva-coated hydroxyapatite beads, and in particular the following 23 strains: *S. thermophilus* YS4 (NCC 2284), *S. thermophilus* Sfi6 (NCC 1971), *S. thermophilus* Sfi13 (NCC 2008), *S. thermophilus* Sfi21 (NCC 2038), *S. thermophilus* Sfi39 (NCC 2130), *S. thermophilus* Sfi42 (NCC 2145), *S. thermophilus* Sfi47 (NCC 2172), *S. thermophilus* S118 (NCC 1529), *S. thermophilus* S119 (NCC 1536), *S. thermophilus* S122 (NCC 1554), *S. thermophilus* S123 (NCC 1561), *S. thermophilus* S126 (NCC 1587), *L. lactis subsp. cremoris* 15 (NCC 92), *L. lactis subsp. cremoris* 25 (NCC 1932), *L. lactis subsp. cremoris* 136 (NCC 2419), *L. lactis subsp. diacetylactis* 8 (NCC 1970), *L. lactis subsp. diacetylactis* 28 (NCC 2057), *L. lactis subsp. diacetylactis* 69 (NCC 2225), *L. lactis subsp. diacetylactis* 80 (NCC 2272), *L. lactis subsp. lactis* 29 (NCC 2211), *L. lactis subsp. lactis* 50 (NCC 2224), *L. lactis subsp. lactis* 54 (NCC 2228), *L. lactis subsp. lactis* 216 (NCC 2484).

The 5 oral strains, *S. sobrinus* OMZ 176, *S. oralis* OMZ 607, *A. naeslundii* OMZ 745, *V. dispar* OMZ 493 and *F. nucleatum* OMZ 596 were obtained from the Institute für Orale Mikrobiologie und Allgemeine Immunologie, University of Zürich and they were cultured in FUM medium in anaerobiosis (GasPackSystem, BBL) at 37°C.

All the strains were stored in glycerol at –20°C and precultured for 14 hours prior to use at their specific optimal temperature; *S. sobrinus* OMZ 176 grew in FUM medium (Neeser *et al.*, Oral Microbiol Immunol, 9, 193-201, 1994), lactococci and streptococci in M17 (Difco) except *S. thermophilus* NCC1529, S119, S122, NCC1561 and S126 that grew in Belliker (prepared by dissolution in 1 l water of 20 g tryptone, 5 g yeast extract, 2.5 g gelatine 5 g dextrose, 5 g sucrose, 5 g lactose, 4 g NaCl, 0.5 g Ascorbic acid, 10 g beef extract).

For the plate counting: *S. sobrinus* OMZ 176 was cultured in Mitis-Salivarius agar (Difco), *S. thermophilus* NCC1529, S119, S122, NCC1561 and S126 in Belliker agar (prepared by adding to the liquid Belliker 15 g of Bacto agar, Difco), and the remaining lactic bacteria strains in M17 agar (Oxoid).

Example 2: PRODUCTION OF A MONOCLONAL ANTIBODY

A monoclonal antibody would be used as a tool to detect *L. lactis subsp. lactis* NCC2211 among 5 oral strains growing together on S-HA discs and forming a biofilm that simulates dental plaque. Therefore the monoclonal antibody was tested against these strains to verify there was no cross-reaction.

To this end, the monoclonal antibody is produced as described by Granato et *al.* "A mouse monoclonal IgE antibody anti-bovine milk lactoglobulin allows studies of allergy in the gastrointestinal tract., Clin. Exp. Immunol., 63, 703-710, 1986.

Example 3: SELECTION OF ADHERENT LACTIC BACTERIA

Attachment to saliva-coated hydroxyapatite beads (S-HA)

To select among the lactic bacteria dairy strains those able to attach to saliva-coated hydroxyapatite beads (S-HA), the procedure previously described by Neeser *et al.* (1994) was used with slight modifications: beads washings were done with 150 µl volumes and Hyamine hydroxide was substituted with Benzethonium hydroxide (Sigma).

Briefly, all the strains were grown to the end of the log phase in FUM except *S. thermophilus* NCC1529, S119, S122, NCC1561 and S126 that were cultured in Belliker. *S. sobrinus* OMZ 176, *L. lactis subsp. lactis* NCC2211, 50

and 54, *S. thermophilus* NCC1529, S119, S122, NCC1561 and S126 grew at 37°C, the remaining lactococci at 30°C and the remaining streptococci at 42°C.

5 mg of hydroxyapatite beads (BDH Chemicals Ltd, Poole, England) were covered with 70 µl clarified saliva obtained from volunteers in the lab and prepared as previously explained (Neeser *et al.*, 1994). Saliva coated beads were kept overnight at 4°C, then washed (first with distilled water and after with HEPES buffer) and finally inoculated with 100 µl of the metabolically labelled bacterial suspension (bacteria had been grown in their medium, supplemented with 10 µCi/ml ^{14}C acetic acid). Adhesion took place during 45 min at 37°C, then unbound bacteria were washed away and the attached cells directly counted in a LKB scintillation counter (type 1219 Rackbeta).

The adhesion percentages are expressed as radioactivity bound to the beads on the total radioactivity added to each well. All measurements were done in triplicate. Table 2 reports the percentages of adhesion to saliva-coated hydroxyapatite beads obtained for several screened strains and for *S. sobrinus* OMZ176 (the reference strain).

STRAIN	% ADHESION (+/-SD)
***S. sobrinus* OMZ 176**	**2.23 +/-0.49**
S. thermophilus Sfi42 (NCC 2145)	0.08 +/-0.02
S. thermophilus Sfi47 (NCC 2172)	0.14 +/-0.04
***S. thermophilus* NCC1529**	**2.89 +/-0.60**
S. thermophilus S119 (NCC 1536)	0.15 +/-0.04
S. thermophilus S122 (NCC 1554)	0.93 +/-0.17
***S. thermophilus* NCC1561**	**2.19 +/-0.50**
S. thermophilus S126 (NCC 1587)	1.19 +/-0.56
L. lactis subsp. diacetylactis 28 (NCC 2057)	1.59 +/-0.17
***L. lactis subsp. diacetylactis* NCC2225**	**1.96 +/-0.40**
L. lactis subsp. diacetylactis 80 (NCC 2272)	1.20 +/-0.35
***L. lactis subsp. lactis* NCC2211**	**2.85 +/-0.85**

Table 2: percentages of adhesion to saliva-coated hydroxyapatite beads obtained for several screened strains

Four strains, *S. thermophilus* NCC1529, *S. thermophilus* NCC1561, *L. lactis subsp. lactis* NCC2211 (hereinafter *L. lactis* NCC2211) and *L. lactis subsp.diacetylactis* NCC2225 showed values close to *S. sobrinus* OMZ 176.

L. lactis NCC2211 and *S. thermophilus* NCC1561 were chosen as the more promising candidates since they grow very well at 37°C, which is the temperature in the mouth, while *L. diacetylactis* NCC2225 has an optimal growth temperature of 30°C. In particular, *L. lactis* NCC2211 cannot grow on sucrose, but it can ferment a wide range of sugars, moreover other oral strain can provide glucose via their invertase.

Adhesion saturation curves

Curves of bound CFU versus CFU inoculated into the well were determined to verify if beads saturation could be obtained. The 50% saturation was directly drawn from the bending point of the curves obtained. The adhesion saturation curves for *S. sobrinus* OMZ 176, *L. lactis* NCC2211 and *S. thermophilus* NCC1561 were determined. They are shown in the Figure 1.

For the three strains the CFU number to be inoculated in the well to get the 50% beads saturation and the corresponding number of bound CFU were directly deduced from the bending point of the curves and are given in the table 3.

	cfu/well	Bound cfu	% adhesion
S. sobrinus OMZ 176	4.00E+07	4.00E+06	10%
L. lactis NCC2211	1.00E+07	9.00E+05	9%
S. thermophilus NCC1561	3.00E+07	2.00E+06	7%

Table 3: Number of CFU to be inoculated per well to get the 50% beads saturation.

Example 4: EFFECT OF CASEINOGLYCOMACROPEPTIDES

The influence of CGMP on the adhesion of *L. lactis* NCC2211 and *S. thermophilus* NCC1561 was studied to verify the possibility of using it to foster the predominance of one of this two strains on the pathogenic ones (namely *S. Sobrinus* OMZ 176. Caseino-glycopeptide (CGMP) and its desialylated derivative (As-CGMP) were obtained from Nestec S. A., Lausanne (for their preparation see Neeser *et al.*, 1994).

The dose-response effect was studied on the adhesion to S-HA beads by inoculating in the well 100 µl of bacterial suspension (CFU/ml corresponding to the 50% beads saturation previously calculated) which contained CGMP or As-CGMP in different concentrations and then performing the adhesion assay as

usual. Concentrations in the range 0.05-3 mg/ml were tested. No previous incubation of the bacteria in presence of CGMP or As-CGMP was done.

In the figure 2, the curves obtained for the three strains by plotting the number of bound cells versus increasing amounts of CGMP are presented, the number of inoculated cells being the one corresponding to the 50% beads saturation formerly calculated for each strain. The strong inhibition observed in the case of *S. sobrinus* OMZ 176 confirms the previous results obtained by Neeser *et al.* (1994) and Schüpbach *et al.* (J. Dent. Res., 75, 1779-1788, 1996).

As evidenced in the figure 2, 0.25 mg/ml produced 50% inhibition of the adhesion of *S. sobrinus* OMZ 176, while more than 2 mg/ml were necessary to have the same effect with *S. thermophilus* NCC1561. CGMP slightly enhances the adhesion of *L. lactis* NCC2211.

As in the case of CGMP, the desyalilated derivative inhibits the adhesion of *S. sobrinus* OMZ 176; only 0.05 mg/ml are needed to produce 50% decrease in the adhesion percentage. As-CGMP does not influence *L. lactis* NCC2211 adhesion, while it slightly fosters the one of *S. thermophilus* NCC1561 (Fig. 3).

Example 5: TOOTHPASTE

Toothpaste is prepared by adding 10^5 cfu/ml of at least one of the lactic bacteria strain CNCM I-1984, CNCM I-1985, CNCM I-1986 or CNCM I-1987 in a lyophilised form, to the following mixture containing:

Cetyl pyridinium chloride	1.65%
Sorbitol (70% soln)	33.0%
Glycerin	25.0%
Sodium carboxymethyl cellulose	2.0%
Sodium fluoride	0.25%
Silica (RP 93)	26.3%
Thickening Silica (Sident 22)	8.1%
Sodium saccharine	0.5%
Poloxamer (Pluronic F108)	3.2%,

This toothpaste is intended for the prophylaxis or the treatment of dental caries, dental plaque and periodontal infection.

Example 6: ICE CREAM

A cream comprising 10.8% lactic fats, 13.5% milk solids (non fat), 0.3% Emulstab® SE30 et 0.3% Emulstab® foam (Grindsted, DK) is prepared, it is then pasteurised at 105°C for 20s, homogenised at 75°C and 300 bar, cooled to 38°C and inoculated with precultures in MRS medium, taken in exponential growth phase, at a rate of 10^7-10^8 cfu/ml of at least one of the lactic bacteria strain CNCM I-1984, CNCM I-1985, CNCM I-1986 or CNCM I-1987. The cream is then fermented for 10 hours at 38°C up to a pH of about 4.5. At the end of the fermentation, sucrose and glucose syrup is added thereto. The composition of the cream is presented in table 4 below.

The mixture is then beaten, cooled to 4°C, stored at 4°C, chilled to a degree of expansion of 95°C by volume.

Ingredients	Composition (kg)	Fats (%)	Non-fat solids (%)	Sucrose (%)	Solids content (%)
Cream (35%)	30,83	10,79	1,54		12,33
Powdered skimmed milk	12,45		11,95		11,95
Emulstab® SE30	0,41				0,37
Emulstab® foam	0,41				0,36
Water	55,91				
Total: cream base	100,00	10,79	13,49	-	25,01
Cream base	74,14	8,00	10,00	-	18,54
Sucrose	22,06			15,00	15,00
Glucose syrup	3,80				3,00
Fermented Ice cream	100,00	8,00	10,00	15,00	36,54

Table 4

Example 7: YOGHURT

5 l MRS culture medium are sterilised for 15 min at 121°C and then inoculated with 5% by volume of an active culture of at least one of the *S.Thermophilus* strain CNCM I-1984, CNCM I-1985 containing approximately 10^9 cfu/ml. After incubation for 8 h at 41°C, a starter containing $4.5.10^8$ cfu/ml is obtained.

5 l reconstituted skimmed milk having a dry matter content of 10%, to which 0.1% yeast extract has been added, are sterilised for 15 min at 121°C and

inoculated with 2% of an active culture of commercial thickening *Streptococcus thermophilus* containing approximately 10^9 cells/ml. After incubation for 4 h at 41°C, a starter containing $4.5.10^8$ cells/ml is obtained.

One batch of whole milk containing 3.7% fats strengthened with 2.5% skimmed milk powder and then pasteurised for 30 min at 90°C is then inoculated with 2% by volume of the starter of at least one of the strain CNCM I-1984 or CNCM I-1985 and 3% by volume of the starter of thickening *Streptococcus thermophilus*. The inoculated milk is stirred, poured into pots and incubated for 4 h at 41°C.

The yoghurt obtained has a good firm and smooth texture and is intended for the health of the mouth.

Claims

1. The use of lactic bacteria that is not part of the resident microflora of the mouth, and that is low acidifying and is capable of adhering directly to the pellicle of the teeth, for the preparation of a composition intended for the prophylaxis or the treatment of dental caries, dental plaque and periodontal infection.

2. The use according to claim 1, wherein the composition is intended for displacing pathogens of the teeth or preventing their attachment.

3. The use according to claim 1 or 2, wherein the lactic bacteria is less acidifying than the pathogenic strains, contributing to a pH in the oral cavity of about 5.5-7.

4. The use according to any of preceding claims, wherein the lactic bacteria is from dairy origin.

5. The use according to any of preceding claims, wherein at least one lactic bacteria is selected from the group consisting of *Streptococcus thermophilus*, *Lactococcus lactis subsp. lactis*, and *Lactococcus lactis subsp. lactis biovar diacetylactis*.

6. The use according to any of preceding claims, wherein at least one lactic bacteria is selected from the group consisting of the strains CNCM I-1984, CNCM I-1985, CNCM I-1986 and CNCM I-1987.

7. The use according to any of preceding claims, wherein the lactic bacteria adheres to the pellicle of the teeth via adhesion factors.

8. The use according to any of preceding claims, wherein the lactic bacteria has been genetically modified to increase its adherence to the pellicle of the teeth and/ or genetically modified to be even less acidifying.

9. The use of lactic bacteria that is not part of the resident microflora of the mouth, for the preparation of a composition intended for the prophylaxis or the treatment of dental caries, dental plaque and periodontal infection, wherein the lactic bacteria is selected from the group consisting of :

- an acidifying lactic bacteria that adheres to the pellicle of the teeth and that has been genetically modified so that it is low acidifying;
- a non adherent lactic bacteria that is low acidifying and that has been genetically modified so that it adheres to the pellicle of the teeth;
- a non-adherent acidifying lactic bacteria that has been genetically modified so that it adheres to the pellicle of the teeth and genetically modified so that it is low acidifying.

10. The use according to claims 8 and 9, wherein the lactic bacteria has been genetically modified so that it adheres to the pellicle of the teeth via adhesion factors and contribute to a pH in the oral cavity of about 5.5-7.

11. The use according to any of preceding claims, wherein the composition is an edible composition comprising an effective quantity of lactic bacteria for the prophylaxis or the treatment of dental caries, dental plaque and periodontal infection.

12. The use according to any of preceding claims, wherein the composition contains at least 10^4-10^9 cfu /g of lactic bacteria.

13. The use according to any of preceding claims, wherein the lactic bacteria is combined with milk, fermented milk, milk derivatives or bacteriocin.

14. The use according to claim 13, wherein the milk derivatives are selected from any form of caseino-glycomacropeptide, micellar casein, fluorinated micellar casein or rennet milk.

15. A composition for the health of the mouth comprising lactic bacteria that is not part of the resident microflora of the mouth and that is low acidifying and that is capable of adhering directly to the pellicle of the teeth.

16. A composition for the health of the mouth according to claim 15, wherein the lactic bacteria has been genetically modified to increase its adherence to the pellicle of the teeth and/ or genetically modified to be even less acidifying.

17. A composition for the health of the mouth according to claim 15 or 16, wherein the lactic bacteria is selected from the group consisting of :

- a) acidifying lactic bacteria that adheres to the pellicle of the teeth and that has been genetically modified so that it is low acidifying;
- a non adherent lactic bacteria that is low acidifying and that has been genetically modified so that it adheres to the pellicle of the teeth;
- a non-adherent acidifying lactic bacteria that has been genetically modified so that it adheres to the pellicle of the teeth and genetically modified so that it is low acidifying.

18. A composition for the health of the mouth according to any of preceding claims, wherein the lactic bacteria has been genetically modified so that it adheres to the pellicle of the teeth via adhesion factors and contributes to a pH in the oral cavity of about 5.5-7.

19. A composition for the health of the mouth according to any of preceding claims, comprising an effective quantity of lactic bacteria for the prophylaxis or the treatment of dental caries, dental plaque and periodontal infection.

20. A composition for the health of the mouth according to any of preceding claims, containing at least 10^4-10^9 cfu /g of lactic bacteria.

21. A composition for the health of the mouth comprising:
    (1) a lactic bacteria that is not part of the resident microflora of the mouth, which is capable of adhering directly to the pellicle of the teeth and contributing to a pH in the oral cavity of about 5.5-7;
    (2) any forms of caseino-glycomacropeptide, micellar casein, fluorinated micellar casein or rennet milk or bacteriocin.

22. A composition for the health of the mouth according to any of preceding claims, comprising at least one lactic bacteria strain selected from the group consisting of *Streptococcus thermophilus*, *Lactococcus lactis subsp. lactis*, and *Lactococcus lactis subsp. lactis biovar diacetylactis*.

23. A composition for the health of the mouth according any of preceding claims, comprising at least one lactic bacteria strain selected from the group consisting of the strains CNCM I-1984, CNCM I-1985, CNCM I-1986, and CNCM I-1987.

24. A method for screening lactic bacteria capable of adhering the teeth, comprising the steps of :

(1) preparing monoclonal antibodies recognising specific surface proteins of a lactic bacteria strain capable of adhering to the teeth, and

(2) screening any lactic bacteria strain by use of the monoclonal antibody of strain capable of adhering to the teeth.

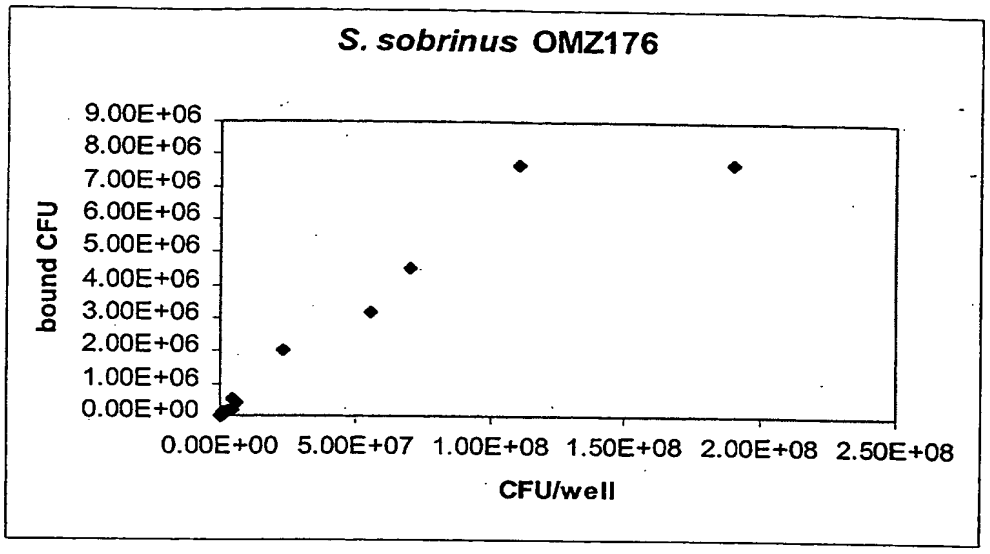

Figure 1a

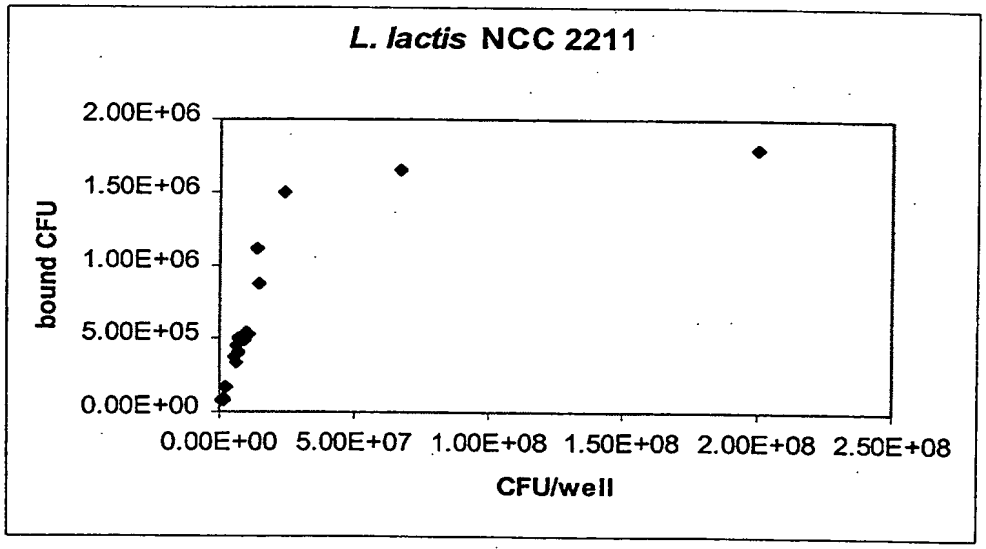

Figure 1b

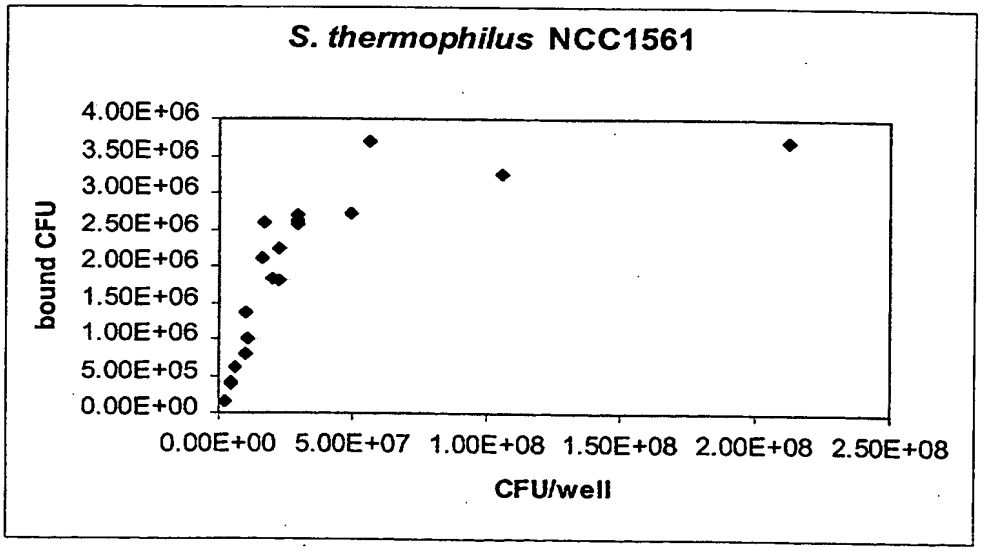

Figure 1c

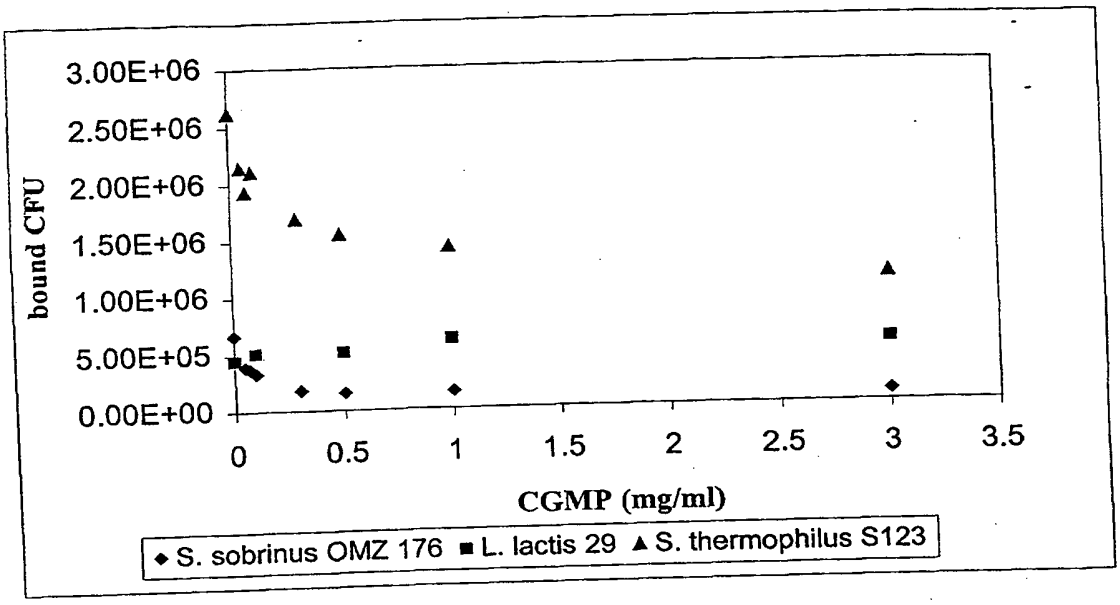

Figure 2. Effect of CGMP on the adhesion to S-HA beads of *S. sobrinus* OMZ 176, *L. lactis* 29 (NCC2211) and *S. thermophilus* S 123 (NCC1561).

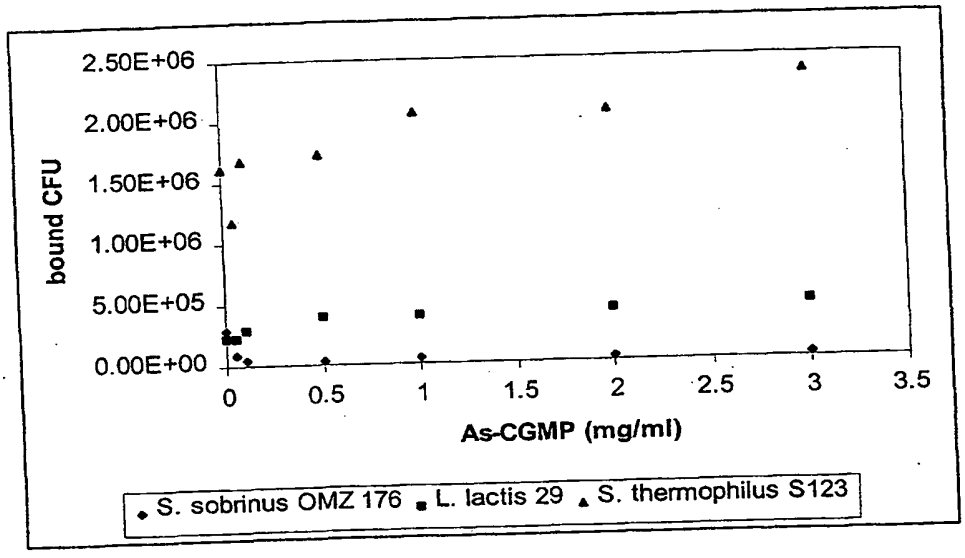

Figure 3. Effect of As-CGMP on the adhesion to S-HA beads of *S. sobrinus* OMZ 176, *L. lactis* 29 (NCC2211) and *S. thermophilus* S123 (NCC1561).

Abstract

The use of lactic bacteria that is not part of the resident microflora of the mouth, and that is low acidifying and is capable of adhering directly to the pellicle of the teeth for the preparation of a composition intended for the prophylaxis or the treatment of dental caries, dental plaque and periodontal infection and compositions prepared therewith.